A Comparison of DNA Extraction Methods using *Petunia hybrida* Tissues

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Extraction of DNA from plant tissue is often problematic, as many plants contain high levels of secondary metabolites that can interfere with downstream applications, such as the PCR. Removal of these secondary metabolites usually requires further purification of the DNA using organic solvents or other toxic substances. In this study, we have compared two methods of DNA purification: the cetyltrimethylammonium bromide (CTAB) method that uses the ionic detergent hexadecyltrimethylammonium bromide and chloroform-isoamyl alcohol and the Edwards method that uses the anionic detergent SDS and isopropyl alcohol. Our results show that the Edwards method works better than the CTAB method for extracting DNA from tissues of *Petunia hybrida*. For six of the eight tissues, the Edwards method yielded more DNA than the CTAB method. In four of the tissues, this difference was statistically significant, and the Edwards method yielded 27–80% more DNA than the CTAB method. Among the different tissues tested, we found that buds, 4 days before anthesis, had the highest DNA concentrations and that buds and reproductive tissue, in general, yielded higher DNA concentrations than other tissues. In addition, DNA extracted using the Edwards method was more consistently PCR-amplified than that of CTAB-extracted DNA. Based on these results, we recommend using the Edwards method to extract DNA from plant tissues and to use buds and reproductive structures for highest DNA yields.

KEY WORDS: CTAB, reproductive and nonreproductive, PCR, agarose gel electrophoresis

INTRODUCTION

The genus *Petunia* belongs to the Solanaceae family of plants and comprises some 30 subspecies.[1] *Petunia* species include annual and perennial herbaceous plants and have been proposed as model organisms for a variety of reasons.[1,2] The flowers of *Petunia* are formed from cymose inflorescences, with each flower bearing five stamens, five sepals, and five petals, arranged in concentric whorls.[3,4] *Petunia hybrida*, commonly known as the Garden Petunia, a hybrid of *Petunia axillaris* and *Petunia integrifolia*, is a commercially valuable ornamental plant.[5,6] In addition, *P. hybrida* has been used as a model to study flavonoid biosynthesis, floral development, and self-incompatibility.[7–12]

Many of the aforementioned studies require DNA extraction from multiple tissue types to be used in assays, such as the PCR. Numerous methodologies have been developed for high-throughput and cost-effective extraction of DNA from plant tissues. These include rapid DNA extraction protocols specifically developed for plants, as well as methods applicable to both plant and animal tissues.[13–19] One of the most commonly used methods to extract DNA from plants uses the ionic detergent cetyltrimethylammonium bromide (CTAB) to disrupt membranes and a chloroform-isoamyl alcohol mixture that separates contaminants into the organic phase and nucleic acid into the aqueous phase.[19] However, many plants contain very high levels of secondary metabolites, including lipids, phenolic compounds, and viscous polysaccharides that can be difficult to remove without further processing, often with organic solvents, such as phenol or other toxic compounds.[13–15] If these contaminants are not removed, then they often inhibit subsequent downstream assays, including PCR.

One alternative to the CTAB DNA extraction method has been developed by Edwards et al.[20] This method uses the anionic detergent SDS to solubilize membranes, followed by precipitation of DNA with isopropyl alcohol. It is a quick, simple, and inexpensive method for extracting DNA from plants, and the DNA can be used directly for PCR amplification without further processing. The Edwards method has been used to extract DNA from various plants, including *Arabidopsis*, soy beans, and corn, and has also been used to extract DNA from processed foods, such as various brands of corn chips.[21,22]

In this paper, we used the CTAB and Edwards methods to extract DNA from different tissues of *P. hybrida* to

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determine: (1) which method yields the highest DNA concentration, (2) which tissue type yields the highest DNA concentration, and (3) which method is better for extracting DNA for PCR amplification.

MATERIALS AND METHODS

Determination of Bud Sizes

All *P. hybrida* plants were purchased from local nurseries in Long Island, New York, USA. Bud development was followed for eight buds, from when the buds were closed and ~1 cm in length to when the flowers opened at anthesis. The cylindrical length of buds (bud sizes) was measured daily, at approximately the same time each day, using Vernier calipers. These data were used to construct a standard curve (Fig. 1) for predicting the age of the buds from subsequent experiments.

Isolation of Genomic DNA

Two methods were used to extract DNA from three different *P. hybrida* plants—a CTAB method and the Edwards method. DNA was extracted from eight plant tissues: (1) young, growing leaf tissue from the apex (apical leaf), (2) mature leaf tissue from the base of the plant (mature leaf), (3) sepals, (4) anthers and pistils from a wilting flower (WF), (5) anthers and pistils from a fresh flower (FF), (6) base of the petals (petals), (7) buds without sepals, 4 days before anthesis (buds, −4 anthesis), and (8) buds without sepals, 1 day before anthesis (buds, −1 anthesis).

CTAB method

This method was modified from Doyle and Doyle.[19] For each of the eight tissues, 50 mg tissue and 100 μl CTAB isolation buffer (2% hexadecyltrimethylammonium bromide, 1.4 M NaCl, 0.2% β2-ME, 20 mM EDTA, 100 mM Tris-HCl, pH 8) were placed into a 1.5-ml microcentrifuge tube, and the tissue was manually crushed for 5 min with a plastic pestle. CTAB isolation buffer (300 μl) was added to each tube, and the tissue was crushed for another 5 min. The sample was incubated at 60°C for 15 min with

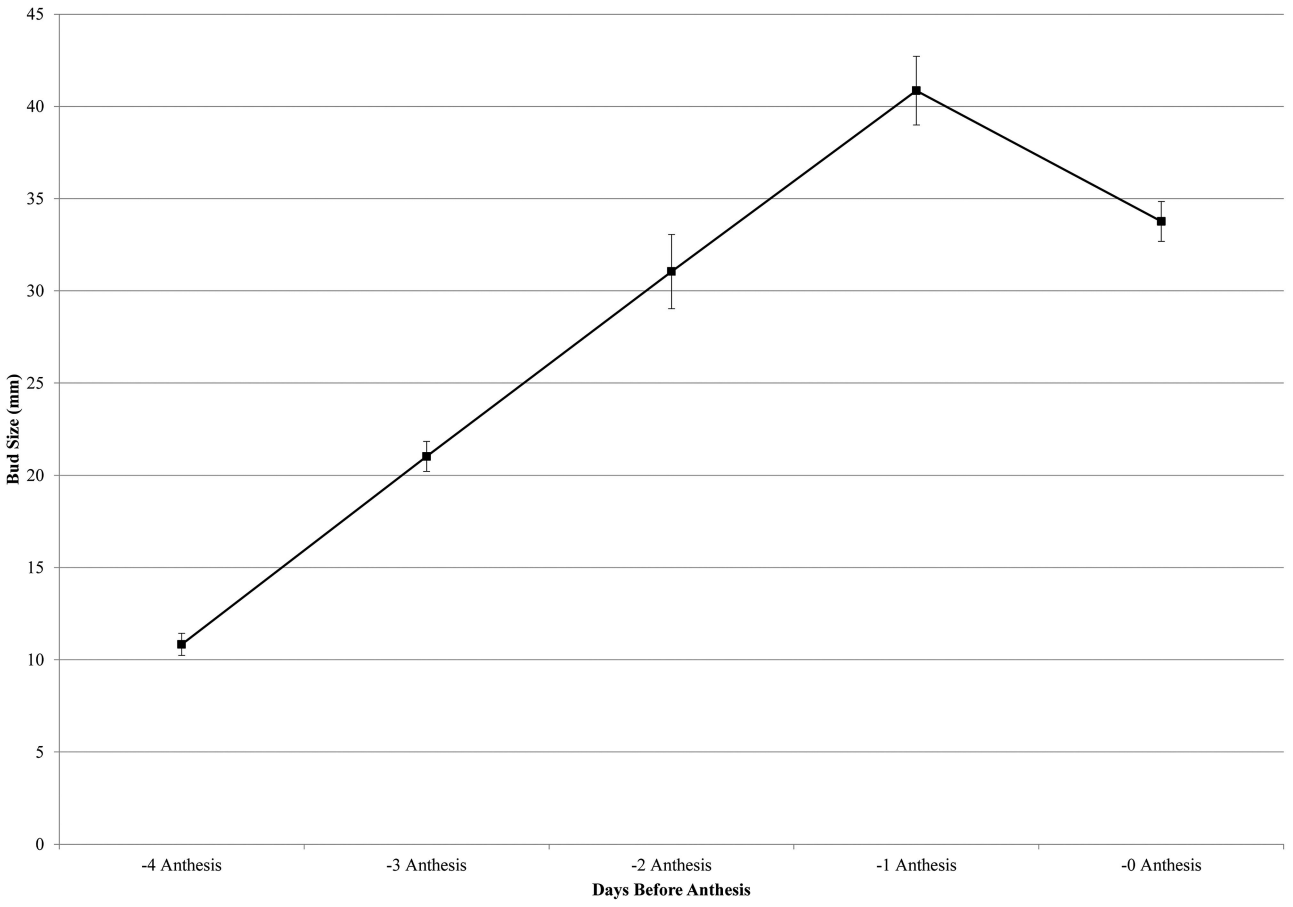

FIGURE 1

Standard curve of *P. hybrida* mean bud sizes. Bud sizes (mm) were measured daily for eight separate buds from 4 days before anthesis (−4 anthesis) until buds opened (−0 anthesis). Mean bud size and standard deviations (brackets) were plotted as a function of time (days before anthesis).

occasional mixing, and then 400 µl chloroform/isoamyl alcohol (24:1 v/v) was added to each sample. The sample was vortexed briefly and then centrifuged for 5 min at 14,000 rpm in a microcentrifuge. The supernatant was transferred to a new 1.5-ml microcentrifuge tube, 300 µl ice-cold isopropanol was added to the tube, and the tube was inverted 5 times to precipitate the nucleic acid. The sample was centrifuged at 14,000 rpm for 5 min in a microcentrifuge, and the supernatant was discarded. The pellet was air-dried for 2 h and then resuspended in 100 µl of 10 mg Ribonuclease A (Sigma R642) in 10 mM Tris, pH 8.0, 1 mM EDTA (TE/RNase A buffer).

Edwards method

This method was modified from Edwards et al.[20] For each of the eight tissues, 50 mg tissue and 200 µl of Edwards buffer (200 mM Tris, pH 8.0, 200mM NaCl, 25 mM EDTA, 0.5% SDS) were placed into a 1.5-ml microcentrifuge tube, and the tissue was manually crushed for 5 min with a plastic pestle. Edwards buffer (300 µl) was added, and the tissue was crushed for another 5 min. The volume was then adjusted to 1000 µl by addition of 500 µl Edwards buffer. The sample was vortexed for 15 s, incubated at 100°C for 10 min, and then centrifuged for 10 min at 2000 rpm in a microcentrifuge. The supernatant (500 µ1) was transferred to a new, 1.5-ml microcentrifuge tube, and the sample was centrifuged again for 10 min at 2000 rpm. The supernatant (400 µl) was transferred to a new, 1.5-ml microcentrifuge tube, and 400 µl ice-cold isopropanol was added. The sample was inverted gently 5 times and incubated at room temperature for 10 min. It was then centrifuged for 10 min at 14,000 rpm in a microcentrifuge, and the supernatant was discarded. The pellet was air-dried for 10 min and then resuspended in 100 µl TE/RNase A buffer.

PCR Amplification and Detection of Amplified DNA

To determine which DNA extraction method was best for PCR amplification of genomic DNA, the DNA extracted from the eight tissues of *P. hybrida* plants was used to PCR amplify a 187-bp region of a plant tubulin gene. The sequence of the forward primer is GGGATCCACTTCATGCTTTCGTCC, and the sequence of the reverse primer is GGGAACCACATCACCACGGTACAT. PCR amplification was performed in 25 µl reactions using PuReTaq Ready-To-Go PCR beads (GE Healthcare, UK; Catalogue #27-9559-01). The PCR bead was first dissolved in 20 µl primer/loading dye mix (6.75 pmol of each primer, 34% sucrose, 0.02% cresol red dye), to which 1 µg tissue DNA was added, and the total volume was adjusted to 25 µl with deionized water. Cycling conditions were 94°C for 5 min; 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, for 32 cycles, and 72°C for 10 min. After PCR amplification, 10 µl of the amplified DNA was separated on a 2% agarose gel, and DNA was visualized by ethidium bromide staining. This protocol was adapted from a method developed at the DNA Learning Center of Cold Spring Harbor Laboratory.[22]

Statistical Analyses

All statistical analyses, including descriptive statistics, Student's *t*-test, and one-way ANOVA, were performed using SPSS 15.1 for Windows (SPSS, IBM, Armonk, NY, USA) and SigmaPlot (version 12; Systat Software, Chicago, IL, USA). Unless otherwise stated, the reported statistical analyses were conducted using SPSS.

RESULTS

In this study, two methods of DNA extraction were compared: the CTAB method and the Edwards method. Additionally, DNA was extracted from eight different tissues of three individual *P. hybrida* plants using both methods, and three replicates were used for each tissue type. In the plant sciences, many investigators choose to extract DNA from reproductive tissues or from tissues that are mitotically active. The rationale is that in both cases, at least some of the cells in the tissues have replicated their DNA and thus, will have higher DNA content. For this reason, we tested tissues that were reproductive in nature or were mitotically active, such as buds, as well as tissues that are not meiotically or mitotically active, such as sepals, petals, and mature leaves. To determine the age of buds, we followed their development over 4 days and found that the increase in bud size was relatively uniform from 4 days before anthesis until 1 day before anthesis (Fig. 1). Interestingly, mean bud size decreased from 1 day before anthesis to anthesis. With the use of a standard curve constructed from these data, the bud ages for buds used in DNA extractions were determined.

Comparison of DNA Yields using the CTAB and the Edwards Methods

The overall mean DNA concentration across all tissues using the CTAB method was 581.5 ± 240.7 µg/µl (n=65) and for the Edwards method, was 930.3 ± 508.5 µg/µl (n=76). There was a statistically significant difference between the two means using a two-tailed *t*-test ($P<0.001$). Table 1 shows mean DNA concentrations in extracts prepared from eight *P. hybrida* tissues using the CTAB method and the Edwards method. For the CTAB method, the mean DNA yields from individual tissues ranged from 341.7 (±97.6) to 897.2 (±110.7) µg/µl and for the Edwards method, 300.0 (±81.0) to 1558.3 (±337.5) µg/µl. The mean DNA concentrations were higher using the Edwards method from all tissues tested, except mature leafs and petals. A one-way ANOVA, followed

TABLE 1

Comparison of mean concentrations of DNA (μg/μl) isolated from reproductive and nonreproductive tissues using the CTAB method and the Edwards method

		CTAB method			Edwards method			
Sample	Tissue	*n*	x̄ (μg/μl)	±SD	*n*	x̄ (μg/μl)	±SD	*P*
1	Apical leaf	8	790.6	130.2	9	852.8	230.0	1.000
2	Mature leaf	9	341.7	97.6	9	302.8	73.4	1.000
3	Sepals	9	452.8	105.7	9	563.9	126.9	0.997
4	Anther/pistil (WF)	3	408.3	104.1	7	1057.1	218.3	<0.001[a]
5	Anther/pistil (FF)	9	575.0	147.4	9	1138.9	283.4	<0.001[a]
6	Petals	9	347.2	138.3	9	300.0	81.0	1.000
7	Buds (−4 anthesis)	9	897.2	110.7	15	1558.3	337.5	<0.001[a]
8	Buds (−1 anthesis)	9	747.2	169.8	9	1277.8	204.4	<0.001[a]

Mean (x̄) DNA concentrations (μg/μl) and SD of DNA isolated from reproductive and nonreproductive tissues using the CTAB method and the Edwards method. Number of replicates for each tissue type (*n*).

[a]*P* values (*P*) generated by SPSS software are shown for one-way ANOVA, followed by Tukey's a posteriori test with statistically significant *P* values indicated. Any *P* value <0.05 is considered as statistically significant.

by Tukey's a posteriori test, showed a statistically significant difference between DNA concentrations obtained using the two extraction methods for the bud and anther/pistil tissues analyzed (Samples 4, 5, 7, and 8), as indicated by the *P* values provided in Table 1. For these samples, DNA concentrations from the Edwards method were always statistically higher. The same was true for two other tissues (Samples 1 and 3), albeit not statistically significant.

A comparison of the range of DNA concentrations obtained from the different tissues is shown in Table 2. For anthers and pistils from a WF and buds −1-day anthesis (Samples 4 and 8, respectively), all replicate extractions using the Edwards method yielded higher DNA concentrations than all replicate extractions using the CTAB method. For example, in Sample 4, the maximum DNA concentration obtained using the CTAB method (525 μg/μl) is lower than the minimum DNA concentration obtained using the Edwards method (800 μg/μl). For anthers and pistils from a FF and buds −4-days anthesis (Samples 5 and 7, respectively), all replicate extractions, except one, yielded higher DNA concentrations using the Edwards method. Comparing the two methods, the range of DNA concentrations was similar for the other four tissues using either extraction method (Samples 1–3 and 6). Taken together, these results show that DNA concentrations were consistently higher in extracts prepared using the Edwards method compared with the CTAB method.

Comparison of DNA Yields from Different Tissues

The tissue that gave the highest DNA concentration for both methods was the buds at 4 days before anthesis (Table 1, Sample 7). Buds at 1 day before anthesis gave the

TABLE 2

Range of DNA concentrations obtained using the CTAB method and the Edwards method

		[DNA] range (μg/μl)	
Sample	Tissue	CTAB method	Edwards method
1	Apical leaf	650–1000	525–1125
2	Mature leaf	250–525	225–425
3	Sepals	325–600	450–825
4	Anther/pistil (WF)	325–525	800–1350[a]
5	Anther/pistil (FF)	350–750	750–1500[b]
6	Petals	175–525	150–425
7	Buds (−4 anthesis)	750–1075	925–1875[b]
8	Buds (−1 anthesis)	525–950	975–1625[a]

DNA yield ranges show minimum and maximum DNA concentrations (μg/μl).

[a]All DNA extraction replicates for this tissue had greater DNA yields using the Edwards method than the CTAB method.

[b]All but one DNA extraction replicate for this tissue had greater DNA yields using the Edwards method than the CTAB method.

second-highest concentration for the Edwards method (Sample 8) but yielded slightly less DNA than apical leaf tissue (Sample 1) for the CTAB method. Reproductive tissue also yielded high DNA concentrations for both methods (Samples 4 and 5) compared with nonreproductive tissue, such as mature leafs, sepals, and petals (Samples 2, 3, and 6, respectively). We believe this is because buds and reproductive tissue possess more actively dividing cells than mature leaves, sepals, and petals and therefore, have a higher DNA content/cell. Based on these results, for maximum DNA yield, we recommend extraction from buds and reproductive structures.

PCR Amplification with Extracted DNA

An important component of any DNA extraction method is its use in downstream applications. Therefore, we compared PCR amplification of a plant tubulin gene using the petunia-tissue DNA extracts. Figure 2 shows a representative agarose gel containing a 187-bp fragment of plant tubulin that was PCR-amplified from DNA of eight petunia tissues (Lanes 1–8). The relative amount of each PCR product can be approximated by comparing the PCR product with the 200-bp DNA fragment of the 100-bp ladder (lane M), which contains 62.5 ng DNA. The upper half of the gel shows PCR products amplified from DNA, extracted using the Edwards method, whereas the lower half of the gel shows PCR products from DNA extracted using the CTAB method.

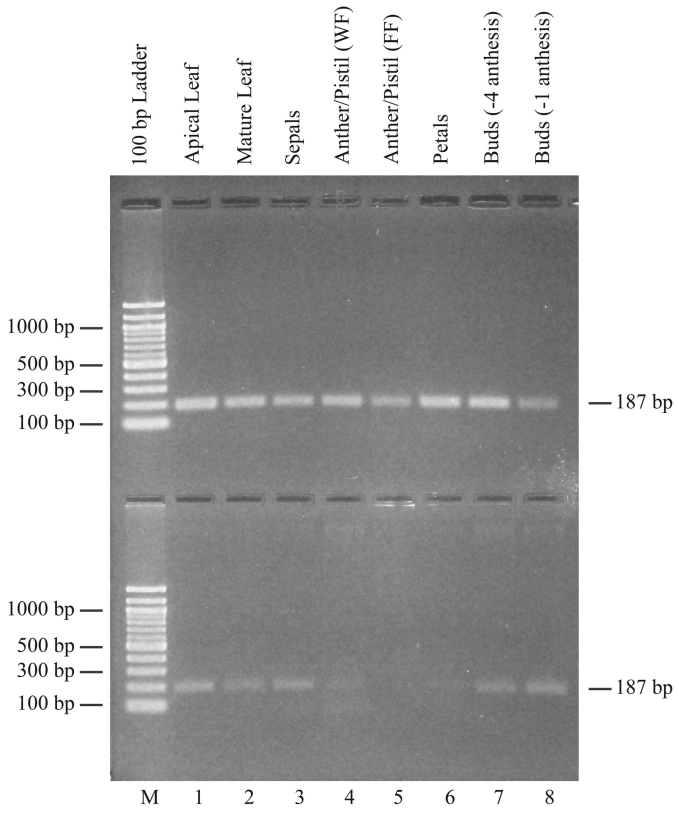

FIGURE 2

Comparison of PCR amplification using genomic DNA purified with the Edwards method or the CTAB method. The Edwards method (upper half of gel) or the CTAB method (lower half of gel) was used to purify genomic DNA from eight tissues of *P. hybrida*. The genomic DNA was used to amplify a 187-bp fragment of a plant tubulin gene, and the PCR products were separated on a 2% agarose gel. The eight tissues used were: (Lane 1) young, growing leaf tissue from the apex (Apical Leaf), (Lane 2) old leaf tissue from the base of the plant (Old Leaf), (Lane 3) Sepals, (Lane 4) anthers and pistils from a WF [Anther/Pistal (WF)], (Lane 5) anthers and pistils from a FF [Anther/Pistal (FF)], (Lane 6) base of the petals (Petals), (Lane 7) buds without sepals, 4 days before anthesis [Buds (−4 anthesis)], and (Lane 8) buds without sepals, 1 day before anthesis [Buds (−1 anthesis)]. Lane M contains a 100-bp DNA ladder.

DISCUSSION

Our results indicate that the Edwards method is more consistent in producing extracts that result in PCR-amplifiable DNA than the CTAB method. First, the overall amount of PCR products for DNA extracted using the Edwards method was similar across the eight tissue types. However, the amount of PCR products was more variable for DNA extracted using the CTAB method—for Lanes 4–6, there is almost no detectable PCR product. PCR amplification using DNA extracted with the CTAB method always showed more variability in the amount of PCR products, although not always with the same anther/pistil and petal tissue samples, as seen in the gel in Fig. 2 (data not shown). Second, the amount of PCR product obtained from each DNA sample extracted using the Edwards method was always greater than or equal to the amount of PCR product obtained using the CTAB method. In the gel in Fig. 2, the amount of all PCR products was greater using the Edwards method than the CTAB method, except for buds −1 day from anthesis (Lane 8), which was approximately equal (compare PCR products in the upper half of the gel with those in the lower half of the gel). Finally, the Edwards method worked better with tissues that were hard to grind, such as anthers (Lanes 4 and 5), as well as tissues that were easy to grind, such as apical leaf (Lane 1).

In conclusion, our results show that the Edwards method works better than the CTAB method for extracting DNA from tissues of *P. hybrida*. The Edwards method extracted more DNA than the CTAB method for the majority of the tissues tested. In addition, DNA extracted using the Edwards method was more consistently PCR-amplified than DNA extracted using the CTAB method. We also found that buds, 4 days before anthesis, yielded the highest DNA concentrations and that in general, buds and reproductive tissue yielded higher DNA concentrations than other tissues. Another advantage of the Edwards

method is that organic solvents are not required for further purification of plant material prior to PCR amplification.

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DISCLOSURES

There are no conflicts of interest.

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